Genome-Wide Association Study of Diabetic Kidney Disease Highlights Biology Involved in Glomerular Basement Membrane Collagen


ABSTRACT

Background Although diabetic kidney disease demonstrates both familial clustering and single nucleotide polymorphism heritability, the specific genetic factors influencing risk remain largely unknown.

Methods To identify genetic variants predisposing to diabetic kidney disease, we performed genome-wide association study (GWAS) analyses. Through collaboration with the Diabetes Nephropathy Collaborative Research Initiative, we assembled a large collection of type 1 diabetes cohorts with harmonized diabetic kidney disease phenotypes. We used a spectrum of ten diabetic kidney disease definitions based on albuminuria and renal function.

Results Our GWAS meta-analysis included association results for up to 19,406 individuals of European descent with type 1 diabetes. We identified 16 genome-wide significant risk loci. The variant with the strongest association (rs55703767) is a common missense mutation in the collagen type IV alpha 3 chain (COL4A3) gene, which encodes a major structural component of the glomerular basement membrane (GBM). Mutations in COL4A3 are implicated in heritable nephropathies, including the progressive inherited nephropathy Alport syndrome. The rs55703767 minor allele (Asp326Tyr) is protective against several definitions of diabetic kidney disease, including albuminuria and ESKD, and demonstrated a significant
association with GBM width; protective allele carriers had thinner GBM before any signs of kidney disease, and its effect was dependent on glycemia. Three other loci are in or near genes with known or suggestive involvement in this condition (BMP7) or renal biology (COLEC11 and DDR1).

Conclusions The 16 diabetic kidney disease–associated loci may provide novel insights into the pathogenesis of this condition and help identify potential biologic targets for prevention and treatment.

The devastating diabetic complication of diabetic kidney disease (DKD) is the major cause of worldwide.1,2 Current treatment strategies at best slow the progression of DKD, and do not halt or reverse the disease. Although improved glycemic control influences the rate of diabetic complications, a large portion of the variation in DKD susceptibility remains unexplained: one third of people with type 1 diabetes (T1D) develop DKD despite adequate glycemic control, whereas others maintain normal renal function despite long-term severe chronic hyperglycemia.3

Though DKD demonstrates both familial clustering4–6 and single nucleotide polymorphism (SNP) heritability,7 the specific genetic factors influencing DKD risk remain largely unknown. Recent genome-wide association studies (GWAS) have only identified a handful of loci for DKD, albuminuria, or eGFR in individuals with diabetes.7–13 Potential reasons for the limited success include small sample sizes, modest genetic effects, and lack of consistency of phenotype definitions and statistical analyses across studies. Through collaboration within the JDRF Diabetes Nephropathy Collaborative Research Initiative, we adopted three approaches to improve our ability to find new genetic risk factors for DKD: (1) assembling a large collection of T1D cohorts with harmonized DKD phenotypes, (2) creating a comprehensive set of detailed DKD definitions, and (3) augmenting genotype data with low frequency and exome array variants.

**METHODS**

**Cohorts and Phenotype Definitions**

The GWAS meta-analysis included up to 19,406 patients with T1D of European origin from 17 cohorts (for study list and details see Supplemental Table 1). All participants gave informed consent and all studies were approved by ethics committees from participating institutions. We defined a total of ten different case-control outcomes to cover the different aspects of renal complications, using both albuminuria and eGFR (Figure 1). Five comparisons (“All versus control (ctrl),” “Micro,” “diabetic nephropathy [DN],” “Macro,” and “ESKD versus macro”) were on the basis of albuminuria, measured by albumin excretion rate (AER) from overnight or 24-hour urine collection, or by albumin-to-creatinine ratio. Two out of three consecutive collections were required (when available) to classify the renal status of patients as either normoalbuminuria, microalbuminuria, macroalbuminuria, or ESKD; for detailed thresholds, see Figure 1. Controls with normal AER were required to have a minimum diabetes duration of 15 years; participants with microalbuminuria/macroalbuminuria/ESKD were required to have minimum diabetes duration of 5, 10, and 15 years, respectively, to exclude renal complications of nondiabetic origins. Two comparisons (“ESKD versus ctrl” and “ESKD versus non-ESKD”) were on the basis of presence of ESKD as defined by eGFR<15 ml/min or dialysis or renal transplant. Two phenotypes (“CKD” and “CKD extreme”) were defined on the basis of eGFR estimated by the CKD Epidemiology Collaboration formula: controls had eGFR=60 ml/min per 1.73 m² for both phenotypes, and ≥15 years of diabetes duration; cases had eGFR<60 ml/min per 1.73 m² for the CKD phenotype, and eGFR<15 ml/min per 1.73 m² or dialysis or renal transplant for the CKD extreme phenotype, and ≥10 years of diabetes duration. For the “CKD-DN” phenotype that combined both albuminuria and eGFR data, controls were required to have both eGFR≥60 ml/min per 1.73 m² and

**Significance Statement**

Although studies show that diabetic kidney disease has a heritable component, searches for the genetic determinants of this complication of diabetes have had limited success. In this study, a new international genomics consortium, the JDRF–funded Diabetic Nephropathy Collaborative Research Initiative, assembled nearly 20,000 samples from participants with type 1 diabetes, with and without kidney disease. The authors found 16 new diabetic kidney disease–associated loci at genome-wide significance. The strongest signal centers on a protective missense coding variant at COL4A3, a gene that encodes a component of the glomerular basement membrane that, when mutated, causes the progressive inherited nephropathy Alport syndrome. These GWAS-identified risk loci may provide insights into the pathogenesis of diabetic kidney disease and help identify potential biologic targets for prevention and treatment.
normoalbuminuria; cases had both eGFR $<45$ ml/min per 1.73 m$^2$ and micro- or macroalbuminuria, or ESKD.

GWAS Genotyping, Quality Control, and Imputation
All study samples underwent genotyping, quality control (QC), and imputation centrally at the University of Virginia. In brief, samples were genotyped on the HumanCore BeadChip array (Illumina, San Diego, CA), which contains approximately 250,000 genome-wide tag SNPs and $>200,000$ exome-focused variants. All samples were passed through a stringent QC protocol. Following initial genotype calling with Illumina software, all samples were recalled with zCall, a calling algorithm specifically designed for rare SNPs from arrays. Variant orientation and position were aligned to hg19 (Genome Reference Consortium Human Build 37, GRCh37). Variant names were updated using 1000 Genomes as a reference. The data were then filtered for low-quality variants (e.g., call rates $<95\%$ and excessive deviation from Hardy–Weinberg equilibrium) and samples (e.g., call rates $<98\%$, sex mismatch, extreme heterozygosity). Principal component analysis was performed separately for each cohort to empirically detect and exclude outliers with evidence of non-European ancestry (see Supplemental Material for full QC details, and Supplemental Figure 1 for trait-specific Manhattan and QQ plots). Genotypes were expanded to a total of approximately 49 million by imputation, using the minimac imputation tool$^{14,15}$ and 1000 Genomes Project (phase 3v5) as a reference.

GWAS Analysis
A genome-wide association analysis was performed for each of the case-control definitions under an additive genetic model, adjusting for age, sex, diabetes duration, study site (where applicable), and principal components. We conducted a second set of analyses, adjusting for body mass index and glycated hemoglobin (HbA1c), which we refer to as our fully adjusted covariate model. Allele dosages were used to account for imputation uncertainty. Inverse-variance fixed effects meta-analysis was performed using METAL and the following filters: INFO score $>0.3$, minor allele count $>10$ in both cases and controls, and presence of variant in at least two cohorts (Manhattan and QQ plots each trait and covariate model presented in Supplemental Figure 1). The X chromosome was similarly analyzed for men and women both separately and...
in a combined analysis, with the exception of using hard call genotypes in place of allele dosages.16 We estimated the percentage of variance explained for all genome-wide significant SNPs across all disease definitions using the McKelvey and Zavoina\textsuperscript{17} pseudo-$R^2$ statistic predicting continuous latent variables underlying binary outcomes.

**Glomerular Basement Membrane Measurement in Renin-Angiotensin System Study**

In brief, the renin-angiotensin system study (RASS) was a double-blind, placebo-controlled, randomized trial of enalapril and losartan on renal pathology among 285 normoalbuminuric, normotensive participants with T1D and normal or increased measured GFR (>90 ml/min per 1.73 m$^2$).\textsuperscript{18} Participants were followed for 5 years with percutaneous kidney biopsy completed prior to randomization and at 5 years. Structural parameters measured by electron microscopy on biopsy included glomerular basement membrane (GBM) width, measured by the electron microscopic orthogonal intercept method.\textsuperscript{18} All RASS participants contributed DNA for genotyping.

**In Silico Replication in SUMMIT Consortium**

The Surrogate Markers for Micro- and Macro-vascular Hard Endpoints for Innovative Diabetes Tools (SUMMIT) consortium included up to 5193 European Ancestry participants with type 2 diabetes (T2D), with and without kidney disease. In silico replication was performed on previously published GWAS on DKD with harmonized trait definitions for seven of our primary T1D analyses: “DN,” “Micro,” “Macro,” “ESKD,” “ESKD versus non-ESKD,” “CKD,” and “CKD-DN” under an additive model, adjusting for age, sex, and duration of diabetes.\textsuperscript{13}

**RNA-Sequencing and Microarray Profiling of Human Kidney Samples from the Pima Cohort**

Kidney biopsy samples from the Pima Indian cohort were manually microdissected into 119 glomerular and 100 tubule-interstitial tissues to generate gene expression profiles.\textsuperscript{19} Expression profiling in the Pima Indian cohort kidney biopsies was carried out using Affymetrix GeneChip Human Genome U133 Array and U133Plus2 Array, as reported previously, and Affymetrix Human Gene ST GeneChip 2.1,\textsuperscript{20,21} and on RNA-seq (Illumina). The libraries were prepared using the ClonTech SMARTSeq v4 Ultra Low Input polyA selection kit. Samples were sequenced on a HiSeq4000 using single-end, 75 bp reads. Mapping to human reference genome GRCh38.7 was performed with STAR 2.5.2b (https://github.com/alexdobin/STAR). For annotation and quantification of mapping results we used cufflinks, cuffquant, and cuffnorm in version 2.2.1 (https://cole-trapnell-lab.github.io/cufflinks/). After mapping and quantification, principal component analysis and hierarchical clustering was used to identify outliers and reiterated until no more outliers could be identified.

**RNA-Sequencing and cis Expression Quantitative Trait Loci Analysis in Human Kidney Samples from University of Pennsylvania Cohort**

Human kidney samples were obtained from surgical nephrectomies for a total of 455 participants with pathologic data and were manually microdissected under a microscope in RNA later for glomerular and tubular compartments (433 tubule and 335 glomerulus samples). The local renal pathologist performed an unbiased review of the tissue section by scoring multiple parameters, and RNA were prepared using RNAeasy mini columns (Qiagen, Valencia, CA) according to manufacturer’s instructions.

Whole kidney,\textsuperscript{22} tubularm and glomerular\textsuperscript{23} expression quantitative trait loci (eQTL) analyses have been described previously. Tubular and glomerular eQTL data sets were generated by 121 samples of tubules and 119 samples of glomeruli, respectively.\textsuperscript{23} The cis window was defined as 1 Mb up- and downstream of the transcriptional start site ($\pm$1 Mb). The whole kidney cis-eQTL (further referred to as just eQTL) data set was generated from 96 human samples obtained from The Cancer Genome Atlas (TCGA) through the TCGA Data portal.\textsuperscript{22}

**Mouse Kidney Single-Cell RNA-Sequencing**

Kidneys were harvested from 4- to 8-week-old male mice with C57BL/6 background and dissociated into single-cell suspension as described in our previous study.\textsuperscript{24} The single-cell sequencing libraries were sequenced on an Illumina HiSeq with 2\times150 paired-end kit. The sequencing reads were demultiplexed, aligned to the mouse genome (mm10) and processed to generate gene-cell data matrix using Cell Ranger 1.3 (http://10xgenomics.com).\textsuperscript{24}

**Genomic Features of Human Kidney**

Human kidney-specific chromatin immunoprecipitation sequencing data can be found at Gene Expression Omnibus under accession numbers GSM621634, GSM670025, GSM621648, GSM772811, GSM621651, GSM1112806, and GSM621638. Different histone markers were combined into chromatin states using ChromHMM.\textsuperscript{25}

**Gene and Gene Set Analysis**

PASCAL and MAGMA (v1.06) gene and pathway scores were conducted on all 20 sets of GWAS summary statistics using default pathway libraries from BioCarta, REACTOME, and KEGG. MAGENTA (v2, July 2011) pathway analysis included 4725 pathways with a minimum of five genes within the gene set for the ten standard adjustment models. We conducted DEPICT individually on all 20 sets of GWAS summary statistics with $P<10^{-5}$ and additional pooled analyses using genome-wide minimum $P$ values from all 20 analyses (ten phenotypes and two covariate models) and 16 analyses of the eight most related phenotypes, which excluded ESKD versus Macro and Micro.
Transcriptome-Wide Association Study
A transcriptome-wide association study (TWAS) of kidney glomeruli and tubules was performed using MetaXcan with default parameters, on the basis of eQTL data for human glomerular and tubular cells.

RESULTS
Phenotypic Comparisons
We investigated a broad spectrum of DKD definitions on the basis of albuminuria and renal function criteria, defining a total of ten different case-control comparisons to cover the different aspects of disease progression (Figure 1). Seven comparisons were on the basis of albuminuria and/or ESKD (including DN, defined as either macroalbuminuria or ESKD), two were defined on the basis of eGFR (used to classify severity of CKD), and one combined both albuminuria and eGFR data (CKD-DN). Each phenotypic definition was analyzed separately in GWAS; to account for the ten definitions each analyzed under two covariate adjustment models, we estimated the total effectively independent tests as 7.4, allowing us to compute a more conservative study-wide significance threshold (P<6.76×10⁻⁹), on the basis of genome-wide significance (P<5×10⁻⁸) and Bonferroni correction for 7.4 effective tests.

Top Genome-Wide Association Results Highlight COL4A3
GWAS meta-analysis included association results for up to 19,406 individuals with T1D of European descent from 17 cohorts for the ten case-control definitions (Supplemental Table 1). We identified 16 novel independent loci that achieved genome-wide significance (P<5×10⁻⁸) in either the minimal or fully adjusted models, in which four lead SNPs also surpassed our more conservative study-wide significance threshold (Figure 2, Manhattan plot; Table 1; Supplemental Figure 2, A–P, regional association and forest plots). None of the loci reaching genome-wide significance have been previously identified in GWAS or candidate gene studies for DKD or closely related traits. All SNPs with minor allele frequency (MAF) >1% explain 2.5% and 3.0% of the total variance (McKelvey and Zavoina pseudo-\(R^2\)) of DN after adjusting for covariates in the minimal and full covariate models, respectively (Supplemental Table 2).

The strongest signal was rs55703767 (MAF=0.21), a common missense variant (G>T; Asp326Tyr) in exon 17 of COL4A3.
Table 1. Loci associated with DKD at study-wide (P<6.76×10⁻⁵, see bolding) and genome-wide (P<5×10⁻⁸) significance

<table>
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<th>SNP</th>
<th>Chr:pos</th>
<th>Effect Allele</th>
<th>Other Allele</th>
<th>EAF</th>
<th>Notable Gene(s)</th>
<th>Phenotype</th>
<th>OR_{min}</th>
<th>P Value_{min}</th>
<th>OR_{full}</th>
<th>P Value_{full}</th>
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<td>All versus ctrl</td>
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<td>Macro</td>
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<td>DDK1 (B)</td>
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<td>44.75</td>
<td>4.99×10⁻⁷</td>
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</table>

Common variants and/or genes with relevant kidney biology are reported in the top half of the table. Uncommon variants (MAF<2%) with no known relevant kidney biology are reported in the bottom half of the table. Genes are annotated as follows: missense variant in the indicated gene (M); intronic, synonymous, or noncoding variant in the indicated gene (G); gene nearest to lead variant (N); gene has relevant kidney (B). Chr, chromosome; pos, position; EAF, effect allele frequency; min, minimally adjusted covariate model; full, fully adjusted covariate model.

COL4A3. This SNP was associated with protection from DN (odds ratio [OR], 0.79; 95% Confidence Interval [95% CI], 0.73 to 0.84; P=5.34×10⁻¹²), any albuminuria (OR, 0.83; 95% CI, 0.79 to 0.88; P=3.88×10⁻¹⁰), the combined CKD-DN phenotype (OR, 0.77; 95% CI, 0.71 to 0.84; P=5.30×10⁻⁹), and macroalbuminuria (OR, 0.78; 95% CI, 0.72 to 0.85; P=9.28×10⁻⁹). Interestingly, we found that rs55703767 in COL4A3 was more strongly associated in men (OR, 0.73; 95% CI, 0.66 to 0.80; P=1.29×10⁻¹¹) than in women (OR, 0.85; 95% CI, 0.76 to 0.94; P=1.39×10⁻³; R_2=1.58×10⁻²). COL4A3 encodes the α3 chain of collagen type IV, a major structural component of the GBM.²⁷

COL4A3 Variation and Kidney Phenotypes

In persons with T1D and normoalbuminuria, GBM width predicts progression to proteinuria and ESKD independently of glycated hemoglobin (HbA1c).²⁸ We examined the influence of the COL4A3 variant on GBM width in 253 RASS18 participants with T1D and normal AER, eGFR (>90 ml/min per 1.73 m²), and BP, who had biopsy and genetic data (Supplemental Table 3). The DKD-protective minor T allele was associated with 19.7 nm lower GBM width (SEM 8.2 nm; P=0.02), with the lowest mean GBM width among TT homozygotes (Figure 3, Supplemental Table 4), after adjusting for age, sex, and diabetes duration, and without detectable interactions with T1D duration or mean HbA1c. Thus, the protective T allele carriers had thinner GBM before any renal complications.

We did not detect any eQTL association between rs55703767 and COL4A3 expression in mouse glomeruli or in human tissues, and thus assume that the variant affects the COL4A3 structure rather than expression levels. Nevertheless in a Pima Indian cohort of 97 participants with DKD with morphometric and expression data from renal biopsies, COL4A3 expression was negatively correlated with the GBM surface density (filtration surface density) (β=−0.27; P=0.02), which is associated with eGFR in DKD in both T1D and T2D.²⁹,³⁰ Furthermore, in 335 microdissected human glomerulus samples, expression of COL4A3 was negatively correlated with glomerulosclerosis, potentially reflecting podocyte depletion in sclerotic glomeruli.
COL4A3 expression in glomeruli, but not in tubules, was also nominally correlated with eGFR (correlation=0.108; \( P=0.05 \); Supplemental Figure 3).

Evidence for Hyperglycemia Specificity

Hyperglycemia promotes the development of diabetic complications. If a genetic variant exerts a stronger effect in the setting of hyperglycemia, (1) it might not be detected in general CKD, (2) it may be detected whether hyperglycemia is conferred by T1D or T2D, (3) its effect may be stronger at higher glycemic strata, and (4) interventions that reduce glycemia may attenuate the association signal. \( \text{COL4A3} \) rs55703767 was not associated with eGFR in a general population sample of 110,517 mainly nondiabetic participants of European ancestry\(^{31} \) (Supplemental Table 5). However, in a smaller cohort of 5190 participants with T2D and DKD phenotypes in the SUMMIT consortium, we detected a directionally consistent suggestive association of \( \text{COL4A3} \) rs55703767 with DN (two-tailed \( P=0.08 \); Supplemental Table 6).

We further stratified the association analyses by HbA1c in the Finnish Diabetic Nephropathy (FinnDiane) Study, a T1D cohort study with extensive longitudinal phenotypic data.\(^{32} \) On the basis of the time-weighted mean of all available HbA1c measurements for each individual, 1344 individuals had mean HbA1c \(<7.5\% \) (58 mmol/mol), and 2977 with mean HbA1c \(\geq7.5\% \). \( \text{COL4A3} \) rs55703767 was nominally significant (\( P<0.05 \)) only in individuals with HbA1c \(\geq7.5\% \) (Figure 4, Supplemental Figure 4, Supplemental Table 7). However, the interaction between HbA1c and \( \text{COL4A3} \) rs55703767 was not significant (\( P=0.83 \)). Upon further examination, the genetic effect was diminished also in the highest HbA1c quartile.

**Table 1**

<table>
<thead>
<tr>
<th>Pheno</th>
<th>( P )</th>
<th>( P ) HbA1c&lt;7.5</th>
<th>( P ) HbA1c&gt;7.5</th>
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</thead>
<tbody>
<tr>
<td>All vs. ctrl</td>
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<td>0.87</td>
<td>0.0018</td>
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<tr>
<td>DN</td>
<td>0.0022</td>
<td>0.659</td>
<td>0.001</td>
</tr>
<tr>
<td>Macro</td>
<td>0.06</td>
<td>0.663</td>
<td>0.0066</td>
</tr>
<tr>
<td>CKD-DN</td>
<td>0.0117</td>
<td>0.973</td>
<td>0.0079</td>
</tr>
</tbody>
</table>

**Figure 3.** Adjusted residuals of GBM width by rs55703767 genotype and sex. Box and whisker plot of residuals of mean GBM width after adjusting for age, sex, and diabetes duration, stratified by GG, GT, or TT genotype at rs55703767, with overlay of individual data points for both women (pink) and men (blue).

**Figure 4.** Association at rs55703767 (\( \text{COL4A3} \)) stratified by HbA1c below or above 7.5%, for the phenotypes reaching genome-wide significance in the combined meta-analysis. Analysis included 1344 individuals with time-weighted mean HbA1c \(<7.5\% \) (58 mmol/mol), and 2977 with mean HbA1c \(\geq7.5\% \) from the FinnDiane study; the individuals had median 19 HbA1c measurements (range 1–129).
 (>9.3%), as the environmental effect of HbA1c seems to overwhelm any potential genetic effects at COL4A3 (test of heterogeneity nonsignificant). In a similar setting of individuals with T2D from the Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) study (n=3226),\textsuperscript{13,33} no difference was observed for COL4A3 rs55703767 between HbA1c strata below or above 7.5% (Supplemental Figure 5). We performed a similar HbA1c stratified analysis in the Diabetes Control and Complications Trial (DCCT), whose participants, all with T1D, continue to be followed in the Epidemiology of Diabetes Interventions and Complications (EDIC).\textsuperscript{34,35} In DCCT-EDIC the effect of COL4A3 rs55703767 was stronger among those recruited in the secondary cohort (mild retinopathy and longer diabetes duration at baseline) who were originally randomized to conventional treatment and therefore had higher HbA1c than the intensive treatment group (Supplemental Table 8). Taken together, these independent lines of evidence strongly suggest that the COL4A3 variant effect on DKD risk is amplified by poor glycemic control.

Other Association Signals

A comprehensive list of all loci that achieved genome-wide significance from either the minimal or the fully adjusted covariate models is reported in Table 1. The fewer covariates required for the minimal model results in improved statistical power because of fewer participants with missing data, whereas the fully adjusted model allows the identification of associations potentially mediated by covariates. Comparison of the adjustment models revealed strong consistency between the two models (Supplemental Figure 6). Table 1 is stratified into two sets of loci: common and/or known kidney biology loci (top half, n=7) and uncommon and no known kidney biology loci (bottom half, n=9). We focus on loci in the top half of the table, common and/or in/near genes with relevant kidney biology.

Two other genome-wide significant signals were near genes encoding proteins related to collagen. Variant rs12615970 (MAF=0.13), located 53 kb downstream of COLEC11, was associated with CKD (OR, 1.31; 95% CI, 1.19 to 1.45; P=9.43×10⁻³), and rs116772905 (MAF=0.011) in exon 14 of DDR1 was associated with microalbuminuria (OR, 3.78; 95% CI, 2.35 to 6.11; P=4.40×10⁻⁸). rs116772905 is in perfect linkage disequilibrium with rs118124843, the lead association with microalbuminuria for this locus under the full adjustment model (taking into account both body mass index and HbA1c), located 29 kb downstream of DDR1 (OR, 3.97; 95% CI, 2.44 to 6.52; P=3.37×10⁻⁸). COLEC11 encodes a collectin protein containing both a collagen-like domain and a carbohydrate recognition domain for binding sugars, and DDR1 encodes the discoidin domain-containing receptor 1, which binds collagens including type IV collagen.

In addition to COL4A3 rs55703767, three other low-frequency variants achieved study-wide significance (P<6.76×10⁻⁹), each associated with microalbuminuria: rs142823282 (MAF=0.017), 22 kb upstream of TAMM41 encoding a mitochondrial translocator assembly and maintenance protein\textsuperscript{36,37} (OR, 6.75; 95% CI, 3.66 to 12.38; P=1.13×10⁻¹⁰); rs144434404 (MAF=0.011), in intron 1 of BMP7 encoding the bone morphogenetic protein 7 previously implicated in DKD\textsuperscript{38} (OR, 6.75; 95% CI, 3.61 to 12.74; P=2.67×10⁻⁸); and rs145681168 (MAF=0.014), in intron 3 of two transcripts of HAND2 antisense RNA 1 (HAND2-AS1; OR, 5.53; 95% CI, 2.90 to 10.53; P=5.40×10⁻⁹) and 50 kb upstream of HAND2, encoding a heart and neural crest derivatives transcription factor.

Two additional common variants achieved genome-wide significance: rs551191707 (MAF=0.122) in PRNCR1 associated with ESKD when compared with macroalbuminuria (OR, 1.70; 95% CI, 1.40 to 2.04; P=4.39×10⁻⁸); and rs61983410 (MAF=0.213) in an intergenic region on chromosome 14 associated with microalbuminuria (full model OR, 0.78; 95% CI, 0.71 to 0.85; P=3.06×10⁻⁸). The remaining eight variants associated with features of DKD had lower allele frequencies (four with 0.01≤MAF≤0.05 and four with MAF<0.01) and did not achieve study-wide significance.

As we had done for COL4A3 rs55703767, we tested whether the associations of the 15 other variants were amplified by hyperglycemia. None of the 15 variants were significantly associated with eGFR in the general population (Supplemental Table 5). In the smaller SUMMIT T2D cohort we were able to interrogate seven loci with comparable trait definitions. The ORs were directionally consistent in six of them (binomial sign test: P=0.06; Supplemental Table 6). In FinnDiane seven of the remaining 15 loci were observed with sufficient frequency (minor allele counts >10) to allow subgroup analysis. Two additional SNPs (rs149641852 in SNCAIP and rs12615970 near COLEC11) were nominally significant (P<0.05) only in individuals with HbA1c ≥7.5%; however, the genotype-by-HbA1c interaction term was nonsignificant (Supplemental Figure 4, Supplemental Table 7).

Variants Previously Associated with DKD

We investigated the effect of variants previously associated at genome-wide significance with renal complications in individuals with diabetes.\textsuperscript{8–13,39} Across the ten subphenotypes in our meta-analysis, we found evidence of association for seven of nine examined loci (P<0.05; Supplemental Figure 7): We replicated two loci that were previously discovered without overlapping individuals with the current study: SCAF8/CNKSR3 rs12523822, originally associated with DKD (P=6.8×10⁻⁴ for “All versus ctrl”); and UMOD rs77924615, originally associated with eGFR in both individuals with and without diabetes (P=5.2×10⁻⁴ for “CKD”).\textsuperscript{31} Associations at the AFF3, RAGMA-MCTP2, and ERBB4 loci, identified in the Genetics of Nephropathy–an International Effort consortium\textsuperscript{12} comprising a subset of studies included in this current effort, remained associated with DKD, although the associations were attenuated in this larger dataset (RAGMA-MCTP2 rs12437854 P=2.97×10⁻⁵; AFF3 rs75583877 P=5.97×10⁻⁴; ERBB4 rs7588550 P=3.53×10⁻⁵; Supplemental Figure 8). Associations were also observed at the CDCA7/SP3 (rs4972593,
P=0.02 for “CKD-DN,” originally for ESKD exclusively in women, and GLRA3 (rs1564939, P=0.02 for “CKD extremes,” originally for AER), but these analyses also include individuals that overlap with the original studies. Apart from the UMOD locus, none of the 63 loci associated with eGFR in the general population were associated with DKD after correction for multiple testing (P<7.0×10^{-4}; Supplemental Figure 9).

Gene and Gene Set Analysis
We conducted gene-level analyses by using two methods that aggregate SNP summary statistics over a gene region while accounting for linkage disequilibrium: MAGMA and PASCAL.40,41 MAGMA identified five genes at a Bonferroni-corrected threshold (P<0.05/18,222 genes tested =2.74×10^{-6}); the collagen gene COL20A1 associated with “CKD extreme” (full model P=5.77×10^{-7}) and “ESKD versus non-ESKD” (full model P=9.53×10^{-7}), SLC46A2 associated with “All versus ctrl” (P=7.38×10^{-7}), SFXN4 associated with “Macro” (full model P=1.65×10^{-7}), GLT5D1 associated with “ESKD versus macro” (P=1.49×10^{-6}), and SNX30 associated with “All versus ctrl” (P=2.49×10^{-6}) (Supplemental Table 9). Although PASCAL did not identify any significant gene-level associations, the five MAGMA-identified genes had P<5.0×10^{-4} in PASCAL (Supplemental Table 10). Both SFXN4 and CBX8 have been reported to be differentially methylated in patients with diabetes with and without nephropathy.42,43

Additionally, we used MAGMA, PASCAL, DEPICT, and MAGENTA to conduct gene-set analysis in our GWAS dataset. The four methods identified 12 significantly enriched gene sets (Supplemental Table 11). One gene set, “negative regulators of RIG-I MDA5 signaling” was identified in two different pathway analyses (MAGMA and PASCAL) of our fully adjusted GWAS of ESKD versus Macro. Several additional related and overlapping gene sets were identified, including “RIGI MDA5 mediated induction of IFN alpha beta pathways,” “TRAF3 dependent IRF activation pathway,” and “TRAF6 mediated IRF activation” (PASCAL) and “activated TLR4 signaling” (MAGENTA). RIG-I, MDA5 and the toll-like receptor TLR4 are members of the innate immune response system that respond to both cellular injury and infection and transduce highly intertwined signaling cascades. These include the signaling molecules TRAF3 and TRAF6, which induce expression of type 1 IFN and proinflammatory cytokines implicated in the progression of DKD.46,47 Specifically, the TLR4 receptor and several of its ligands and downstream cytokines display differential levels of expression in DKD renal tubules versus normal kidneys and versus non-DKD controls, and TLR4 knockout mouse are protected from DKD and display marked reductions in interstitial collagen deposition in the kidney.49 Other pathways of interest include “other lipid, fatty acid and steroid metabolism,” “nitric oxide signaling in the cardiovascular system,” and “TNF family member,” with both nitric oxide and TNF-α implicated in DKD,50,51,52

Expression and Epigenetic Analyses
We interrogated gene expression datasets in relevant tissues to determine whether our top signals underlie eQTL. We first analyzed genotype and RNA-sequencing gene expression data from 96 whole human kidney cortical samples and micro-dissected human kidney samples (121 tubule and 119 glomerular samples) from participants of European descent without any evidence of renal disease (Supplemental Figure 10). No findings in this data set achieved significance after correction for multiple testing. In the GTEx and eQTLgen datasets, COL4A3 rs55703767 had a significant eQTL (P=5.63×10^{-38}) with the MFF gene in blood, but is most likely due to modest LD with other nearby strong eQTLs in the region. rs118124843 near DDR1 and VARS2 had multiple significant eQTLs in blood besides VARS2 (P=1.71×10^{-5}; Supplemental Table 12). Interestingly, rs142823282 near TAMM41 was a cis-eQTL for PPARG (P=4.60×10^{-7}), a transcription factor regulating adipocyte development and glucose and lipid metabolism; PPARγ agonists have been suggested to prevent DKD.53

To ascertain the potential functional role of our top non-coding signals, we mined chromatin immunoprecipitation sequencing data derived from healthy adult human kidney samples.52 SNP rs142823282 near TAMM41 was located close to kidney histone marks H3K27ac, H3K9ac, H3K4me1, and H3K4me3, suggesting that this is an active regulator of TAMM41 or another nearby gene (Supplemental Figure 11). Interestingly, in recent work we have shown that DNA methylation profiles in participants with T1D with/without kidney disease show the greatest differences in methylation sites near TAMM41.54

To establish whether the expression of our top genes shows enrichment in a specific kidney cell type, we queried an expression dataset of approximately 50,000 single cells obtained from mouse kidneys. Expression was detected for six genes in the mouse kidney atlas: three (COL4A3, SNCAIP, and BMP7) were almost exclusively expressed in podocytes (Figure 5), supporting the significant role for podocytes in DKD.

Gene expression levels in kidneys in cases versus controls were predicted with TWAS on the basis of the GWAS summary statistics and eQTL data of kidney glomeruli and tubuli. While none of the genes survived correction for multiple testing, analysis suggested 18 genes with differential expression in cases and controls with P<1×10^{-4}, including the NPNT, PRR2C, and VPS33B genes (Supplemental Table 13). NPNT encodes for nephronectin, an extracellular matrix protein on GBM. Knocking out NPNT or decreasing NPNT expression levels have been shown to induce podocyte injury related to GBM.55 On the contrary, TWAS predicted higher NPNT expression within DN cases versus normal AER. In line with our TWAS finding, NPNT is significantly upregulated in glomeruli of DN mouse model versus nondiabetic mouse (P=6.4×10^{-4}; fold change 1.3, in top 2%, accessed through www.neoproseq.org).56 Furthermore, a variant near PRR2C was recently associated with albuminuria in the UK Biobank, and rare mutations in VPS33B gene cause arthrogryposis, renal
dysfunction, and cholestasis-1 syndrome involving proximal–tubular dysfunction and usually death by 1 year of age.58

DISCUSSION

Our genome-wide analysis of 19,406 participants with T1D identified 16 genome-wide significant loci associated with DKD, four of which remained significant after a conservative correction for multiple testing. Four of the 16 genome-wide significant signals are in or near genes with known or suggestive biology related to renal function/collagen (COL4A3, BMP7, COLEC11, and DDR1), but this is the first time that naturally occurring variation (MAF >1%) in these loci has been associated with DKD. Our most significant signal was a protective missense variant in COL4A3, rs55703767, reaching both genome-wide and study-wide significance with multiple definitions of DKD. Moreover, this variant demonstrated a significant association with GBM width such that protective allele carriers had thinner GBM before any signs of kidney disease, and its effect was dependent on glycemia. COL4A3, with COL4A4 and COL4A5, make up the so-called “novel chains” of type IV collagen,59 which together play both structural and signaling roles in the GBM. Specifically, COL4A3 is known to bind a number of molecules including integrins, heparin, and heparin sulfate proteoglycans, and other components of the GBM, such as laminin and nidogen. These interactions mediate the contact between cells and the underlying collagen IV basement membrane, and regulate various processes essential to embryonic development and normal physiology, including cell adhesion, proliferation, survival, and differentiation. Dysregulation of these interactions has been implicated in several pathologic conditions, including CKD.60

Mutations in COL4A3 are responsible for the autosomal recessive form of Alport syndrome, a progressive inherited nephropathy, as well as benign familial hematuria, characterized by thin (or variable width) GBM, and thought to be a milder form of Alport syndrome.61 Furthermore, mutations in COL4A3 have also been identified in patients with FSGS, often leading to proteinuria and renal failure. Some of these patients with FSGS presented with segmental GBM thinning.62 Of note, the common rs55703767 (COL4A3 Asp326Tyr) variant, protecting from DKD, was also associated with thinner GBM in individuals with diabetes but without renal complications, a feature that seems to be beneficial in the context of diabetes. The rs55703767 SNP is predicted to alter the third amino acid of the canonical triple-helical domain sequence of Glycine (G)-X-Y (where X and Y are often proline [P] and hydroxyproline [Y], respectively) from G-E-D (D=Aspartic) to G-E-Y,63 potentially affecting the structure of the collagen complex. In addition, a recent study of
candidate genes involved in renal structure reported rs34505188 in COL4A3 (not in linkage disequilibrium with rs55703767, r²<0.01) to be associated with ESKD in black Americans with T2D (MAF=2%; OR, 1.55; 95% CI, 1.22 to 1.97; P=5×10⁻⁴). Together with the trend toward association we have seen in SUMMIT and the glycemic interaction we have reported here, these findings suggest variation in COL4A3 may be associated with DKD in T2D as well.

Given its association as a protective SNP, we can speculate that the rs55703767 variant may confer tensile strength or flexibility to the GBM, which may be of particular relevance in the glomerular hypertension associated with DKD. Alternatively, COL4A3 may regulate the rates of production and/or turnover of other GBM components, affecting GBM width changes in diabetes. How these effects might confer protection in a manner dependent on ambient glucose concentrations is unknown. Future mechanistic studies will be required to determine the precise role of this variant in DKD; elucidation of its interaction with glycemia in providing protection might be relevant to other molecules implicated in diabetic complications.

In keeping with the collagen pathway, the synonymous exonic variant rs118124843, which reached genome-wide significance for the “Micro” phenotype, is located near DDR1, the gene encoding the discoidin domain-containing receptor 1. On the basis of chromatin conformation interaction data from Capture HiC Plotter,⁶⁵ the rs118124843 containing fragment interacts with six gene promoter regions, including DDR1, suggesting that the variant regulates DDR1 expression across multiple tissues (Supplemental Table 12). DDR1 is a collagen receptor⁶⁶ shown to bind type IV collagen,⁶⁷ and is highly expressed in kidneys, particularly upon renal injury.⁶⁸ Upon renal injury, Ddr1-deficient mice display lower levels of collagen,⁶⁹ decreased proteinuria, and an increased survival rate compared with wild-type controls,⁷⁰ with Ddr1/Col4a3 double-knockout mice displaying protection from progressive renal fibrosis and prolonged lifespan compared with Col4a3 knockout mice alone.⁶⁹ Thus, through its role in collagen binding, DDR1 has been suggested as a possible therapeutic target for kidney disease.⁶⁹

The association of rs12615970, an intronic variant on chromosome 2 near the COLEC11 gene, met genome-wide significance for the CKD phenotype, as well as nominal significance for multiple albuminuria-based traits. The rs12615970 containing fragment was found to interact with COLEC11, ALLC, and ADI1 transcription start sites in chromatin conformation data on GM12878 cell line (Supplemental Table 12).⁵⁵,⁷¹ Collectin-11 is an innate immune factor synthesized by multiple cell types, including renal epithelial cells with a role in pattern recognition and host defense against invasive pathogens, through binding to fructose and mannose sugar moieties.⁷²,⁷³ Mice with kidney-specific deficiency of COLEC11 are protected against ischemia-induced tubule injury because of their reduced complement deposition,⁷⁴ and mutations in COLEC11 have been identified in families with 3MC syndrome, a series of rare autosomal recessive disorders resulting in birth defects and abnormal development, including kidney abnormalities.⁷⁵

The intronic variant rs144434404, associated at study-wide significance with the microalbuminuria phenotype, resides within the bone morphogenetic protein 7 (BMP7) gene. BMP7 encodes a secreted ligand of the TGF-β superfamily of proteins. Developmental processes are regulated by the BMP family of glycosylated extracellular matrix molecules via serine/threonine kinase receptors and canonical Smad pathway signaling. Coordinated regulation of both BMP and BMP-antagonist expression is essential for developing tissues, and changes in the levels of either BMP or BMP-antagonists can contribute to disease progression such as fibrosis and cancer.⁷⁶ BMP7 is required for renal morphogenesis, and Bmp7 knockout mice die soon after birth, because of reduced ureteric bud branching.⁷⁷–⁷⁹ Maintenance of Bmp7 expression in glomerular podocytes and proximal tubules of diabetic mice prevents podocyte loss and reduces overall diabetic renal injury.⁸⁰ More recently, we have identified a mechanism through which BMP7 orchestrates renal protection through Akt inhibition, and highlights Akt inhibitors as potential antifibrotic therapeutics.⁸¹ It is also noteworthy that the BMP7 antagonist grem-1 is implicated in DKD,⁸²–⁸³ and gremlin has been implicated as a biomarker of kidney disease.⁸⁴

Strengths of this analysis include the large sample size, triple that of the previous largest GWAS; the uniform genotyping and QC procedures; standardized imputation for all studies (1000 Genomes reference panel); the inclusion of exome array content; the exploration of multiple standardized phenotype definitions of DKD; and supportive data from various sources of human kidney samples. Several of the loci identified have known correlations with kidney biology, suggesting that these are likely true associations with DKD. However, we acknowledge a number of limitations. First, nine variants have low MAF and were driven by only two cohorts, indicating that further validation will be required to increase confidence in these associations. Second, seven variants were significantly associated with microalbuminuria only, a trait shown to be less heritable in previous studies. We included these loci to maximize comprehensiveness in reporting novel DKD associations. Replication in independent samples and functional confirmation is required to validate all of these loci. Although the gene-level, gene set, and pathway analyses had limited power, these analyses identified several additional potential DKD loci and pathways, some with relevance to kidney biology, that require further follow-up. Finally, although we included only controls with a minimum diabetes duration of 15 years, we cannot fully rule out that some of the controls would progress to DKD in the future, as the improvements in diabetes treatment in the past decades have postponed the onset of complications. We also excluded cases with short diabetes duration to avoid renal complications that might be due to other causes. These phenotypic definitions were meant to overcome the limitation that in clinical practice kidney biopsy specimens are rarely taken from individuals with diabetes to verify the
diagnosis. As for any late-onset disease, these challenges in phenotypic definition may have reduced our power to detect additional associations. We note, however, that this relatively small degree of contamination would lead to loss of power and increased type 2 error rather than false positive findings; therefore, it does not undermine the robustness of the associations reported here.

Diabetic complications are unquestionably driven by hyperglycemia and partially prevented by improved glycemic control in both T1D and T2D, but there has been doubt over what contribution, if any, inherited factors contribute to disease risk. In line with previous genetic studies, this study with a markedly expanded sample size identified several loci strongly associated with DKD risk. These findings suggest that larger studies, aided by novel analyses and including T2D, will continue to enhance our understanding of the complex pathogenesis of DKD, paving the way for molecularly targeted preventive or therapeutic interventions.

ACKNOWLEDGMENTS

Conceptualization: Godson, Maxwell, Groop, Hirschhorn, and Florcz; Formal analysis: Salem, Cole, Sandholmen, Valo, Di Liao, Cao, Pezzolesi, Smiles, Qiu, Nair, Park, Liu, Menon, Kang, R. Klein, B.E. Klein, Canty, Paterson, Chen, van Zuydam, and Onengut-Gumuscu; Investigation: Pezzolesi, Smiles, Skupien Qiu, Nair, Haukka, Mcknight, Snell-Begeon, Maahs, Maxwell, Paterson, Forsblom, Ahlyqvist, Ahluwalia, Lajer, Boustanly-Kari, Kang, Maceastroni, Tregouet, Gyorgy, Bull, Palmer, Stechemesser, Paulweber, Weigtasser, Rovite, Pirags, Prakapiene, Radzveiciene, Verkauskiene, Panduru, Groop, McCarthy, Gu, Mollsten, Falhammar, Brismar, Rossing, Costacou, Zerbini, Marre, Hadjadi, Forsblom, Chen, and Onengut-Gumuscu; Resources: Smiles, Harjutsalo, McKnight, Nelson, Caramori, Maurer, Gao, Snell-Begeon, Maahs, Guo, Miller, Maxwell, Paterson, Chen, and Onengut-Gumuscu; Data curation: Hiraki, Di Liao, Cao, Smiles, Harjutsalo, Skupien, McKnight, R. Klein, B.E. Klein, Lee, Gao, Maurer, Caramori, Snell-Begeon, Maahs, Guo, Miller, Maxwell, Chen, and Onengut-Gumuscu; Writing, original draft: Salem, Todd, Sandholmen, Cole, Brennan, Andrews, Doyle, Hughes, Hiraki, Paterson, and Godson; Writing, review and editing: Salem, Todd, Sandholmen, Cole, Brennan, Andrews, Doyle, Hughes, Hiraki, Paterson, Godson, Qiu, Park, Skupien, Maurer, Caramori, de Boer, Martin, McKnight, McKay, Maxwell, Susztak, McCarthy, Groop, Rich, Forsblom, Hirschhorn, and Florcz; Visualization: Salem, Todd, Sandholmen, Cole, Pezzolesi, Qiu., Di Liao, Cao, Park, Skupien, R. Klein, B.E. Klein, Lee, McKnight, McKay, Maxwell, and Paterson; Project administration: Todd; Supervision: Paterson, Krolewski, Groop, Godson, Maxwell, Colhoun, Rich, Kretzler, Susztak, Hirschhorn, and Florcz; Funding acquisition: Paterson, Krolewski, Florcz, and Hirschhorn. All authors approved the final version of the manuscript.

AusDiane acknowledges Dr. Bernhard Baumgartner from Department of Medicine, Diakonissen-Wehrle Hospital, Salzburg, Austria. LatDiane acknowledges the following doctors and researchers: A. Bogdanova, D. Grikmane, I. Care, A. Fjodorova, R. Graudina, A. Valterda, D. Teterovska, I. Dziviete-Krišāne, I. Kirilova, U. Lauga-Turiņa, K. Guldner, D. Seismu, N. Fokina, S. Steina, I. Jaunoziola, I. Balcerie, U. Gailiša, E. Menise, S. Broka, L. Akmene, N. Kapla, I. Nagaičeva, A. Petersons, A. Lejinieks, I. Konrāde, I. Salna, A. Dekante, A. Grāmatniece, A. Salina, A. Sīlda, V. Mēsečko, V. Mīhejeva, K. Kudrjavceva, I. Marksa, M. Čirse, D. Zeme, S. Kalva-Vaivode, J. Klovins, L. Nikitina-Załge, S. Skrebinska, Z. Džerve, and R. Mallons. LitDiane acknowledges Drs. Jurate Lasiene, Prof. Dzilda Velickiene, Dr. Vladmiras Petrenko and nurses from the Department of Endocrinology, Hospital of Lithuanian University of Health Sciences. RomDiane acknowledges the following doctors and researchers: M. Anghel, DM Cheta, BA Cimpoaca, D. Cimponeriu, CN Cozo, N. Danudo, D. Dobrin, I Duta, AM Frentescu, C. Ionescu-Tirgoviste, R. Ionica, R. Lichiardependol, A. Opree, PM Panduru, A. Pop, P. Pop, R. Radescu, S. Radu, M. Robu, C. Serafincneam, M. Stavarachi, O Tudose from the N.C Paulescu National Institute for Diabetes Nutrition and Metabolic Diseases from Bucharest; and M. Bacu, D. Clenciu, C. Graunteama, M. Ioana, E. Mota, and M. Mota from Craiova Emergency County Hospital. SDR: Thanks to Maria Sterner and Malin Népina for GWAS genotyping in the Scanni Diabetes Registry. The FinnDiane study thanks M. Parkkonen, A. Sandelin, A-R. Salonen, T. Soppela, and J. Tuomikangas for skillful laboratory assistance in the FinnDiane study. We also thank all the participants of the FinnDiane study and gratefully acknowledge all the physicians and nurses at each centre involved in the recruitment of participants (Supplemental Table 14). GoDARTS: We are grateful to all the participants in this study, the general practitioners, the Scottish School of Primary Care for their help in recruiting the participants, and to the whole team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists, and nurses. The study complies with the Declaration of Helsinki. We acknowledge the support of the Health Informatics Centre, University of Dundee for managing and supplying the anonymised data and NHS Tayside, the original data owner. For a full list of SUMMIT consortium members, see Supplemental Table 15.

The views expressed in this article are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

Data and Software Availability

GWAS summary statistics for all ten DKD phenotypes and two adjustment models are available for download at the AMP-Type 2 Diabetes Knowledge Portal (http://www.type2diabetesgenetics.org informational/data), under “JDRF Diabetic Nephropathy Collaborative Research Initiative GWAS” datasets. Individual-level genotype data cannot be shared for all cohorts because of restrictions set by study consents and by European Union and national regulations regarding individual genotype data.

DISCLOSURES

We acknowledge the following conflicts of interest: Groop has received investigator-initiated research grants from Eli Lilly and Roche, is an advisory board member for Abbott, AstraZeneca, Boehringer Ingelheim, Cebix, Eli Lilly, Janssen, Medscape, Merck Sharp & Dohme, Novartis, Novo Nordisk
Supplemental Material

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2019030218/-/DCSupplemental.

Supplemental Table 1. Cohorts contributing to analyses.

Supplemental Table 2. Pseudo-$R^2$ of all SNPs across all GWAS as calculated by the McKelvey and Zavoina method.

Supplemental Table 3. Characteristics of RASS participants. Categorical variables display counts and percentage. Continuous values are mean±SD.

Supplemental Table 4. Multivariate analysis of association between rs55703767 and GBM width.

Supplemental Table 5. Look-up of the lead loci in GWAS on eGFR in the general population (Gorski et al.13).

Supplemental Table 6. Look-up of the lead loci in GWAS in the SUMMIT consortium (van Zuydam et al.13).
Supplemental Table 7. Association at lead loci stratified by HbA1c<7.5%.
Supplemental Table 8. Association of rs55703767 with DN in DCC/EDIC subgroups.
Supplemental Table 9. Significant (P<0.05/18,222 genes tested =2.74×10−6) gene level associations with DKD in MAGMA.
Supplemental Table 10. Top nominally significant gene-level associations (P<1.0×10−5) with DKD in PASCAL.
Supplemental Table 11. Significant gene set and pathway analysis results.
Supplemental Table 12. eQTL associations and chromatin conformation interactions for the lead SNPs.
Supplemental Table 13. TWAS results with P<1×10−4.
Supplemental Table 14. Physicians and nurses at health care centers participating in the collection of FinnDiane patients.
Supplemental Table 15. Members of the SUMMIT consortium.
Supplemental Figure 1. Manhattan and QQ plots for each case-control definition and covariate model (minimal and full).
Supplemental Figure 2. Regional chromosomal location plots and forest plots by cohort of newly discovered DKD associations.
Supplemental Figure 3 (S2). Correlation of expression of COL4A3 with degree of fibrosis and eGFR in microdissected kidney samples.
Supplemental Figure 4 (S3). Genotype–phenotype associations at the lead loci when stratified by mean HbA1c <7.5% in the FinnDiane study.
Supplemental Figure 5 (S4): Genotype–phenotype associations at the lead rs55703767 (COL4A3) locus when stratified by mean HbA1c <7.5% in up to 3226 individuals with T2D from the GoDARTS.
Supplemental Figure 6 (S6). Fishplots comparing significance and directionality between minimal and fully adjusted models for each of the ten phenotype definitions.
Supplemental Figure 7 (S5). Association at previously reported loci (P<5×10−8) for renal complications in individuals with diabetes.
Supplemental Figure 8 (S7). Forest plots of the associations at the previously reported lead loci from the GENIE consortium with largely overlapping studies.
Supplemental Figure 9 (S7). Meta-analysis results for the loci that have previously been associated with DKD, or with eGFR or AER in the general population.
Supplemental Figure 10 (S8). eQTL analysis in microdissected tubule samples.
Supplemental Figure 11 (S9). Functional annotation of TAMM41.
Supplemental Appendix 1. Cohort descriptions and references.

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See related editorial, “Biologic Underpinnings of Type 1 Diabetic Kidney Disease,” on pages 1782–1783.

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